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## TITLE:

Claudin k is specifically expressed in myelin during development of the nervous system and after regeneration of the optic nerve in adult zebrafish.

## AUTHORS:

Jolanda Münzel<sup>1, 4, 5, ‡</sup>, Karin Schaefer<sup>2, †‡</sup>, Barbara Obirei<sup>2</sup>, Elisabeth Kremmer<sup>3</sup>, Christian Brösamle<sup>2 # \*</sup>, Edward A. Burton<sup>4</sup>, Veronika Kuscha<sup>5</sup>, Catherina G. Becker<sup>5</sup>, Anna Williams<sup>6 \*</sup>, Thomas Becker<sup>5 \*</sup>

## ADDRESSES:

<sup>1</sup> Centre for Clinical Brain Sciences, University of Edinburgh, United Kingdom

<sup>2</sup> SFB596, Department of Biochemistry, Ludwig Maximilian University Munich, Germany

<sup>3</sup> SFB 596, Helmholtz-Zentrum München, Germany

<sup>4</sup> Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, Philadelphia, USA

<sup>5</sup> Centre for Neuroregeneration, University of Edinburgh, United Kingdom

<sup>6</sup> Edinburgh Centre for MS research, Centre for Regenerative Medicine, University of Edinburgh, United Kingdom

<sup>†</sup> current address: Helmholtz-Zentrum München, Munich Germany

<sup>#</sup> current address: wtb wissenschaftliche texte brösamle, Ismaning, Germany

<sup>‡</sup> Equal contributions

<sup>\*</sup> Joint senior authors

## ABSTRACT:

In recent years, the zebrafish has become an important model organism to study the formation and function of myelin during development and after lesion of the adult CNS. Here we describe *claudin k*, a novel myelin-associated gene in zebrafish, and determine its expression during development and adult optic nerve regeneration. We find Claudin k protein in autotypic tight junctions of oligodendrocytes and myelinating Schwann cells. MRNA Expression starts in the hindbrain at 2 days post-fertilisation and protein expression can be detected after 3 days (protein) and throughout adulthood. Claudin k is also expressed in the adult retinal optic axon layer, which contains only non-compacted myelin sheaths. After a crush of the optic nerve, Claudin k expression is first reduced and then recovers within 4 weeks post-lesion, concomitant with optic nerve myelin de- and re-generation. A *claudin k*:GFP reporter line was generated for live observation of myelination and visualization of myelinating cells, such as changes from simple to complex morphology of oligodendrocytes during regeneration. Thus, *claudin k* is a novel myelin-associated gene expressed by oligodendroglial cells and Schwann cells from early stages of myelination in zebrafish development and regeneration to adulthood, suggesting important functions in myelin formation and maintenance. Antibodies directed against Claudin k and the *claudin k*:GFP reporter line represent valuable tools to visualize and study myelination *in vivo*.

## INTRODUCTION:

Developmental myelination by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) leads to fast, saltatory action potential conduction at the nodes of Ranvier (reviewed in Sherman and Brophy, 2005). In order to increase our understanding of myelination, we have identified a new specific marker of myelinating cells in a highly accessible model vertebrate, the zebrafish (*Danio rerio*).

The zebrafish now has taken a firm place in biological research to study development and has become an established organism to model human diseases (examples reviewed in Newman et al., 2011; Bandmann and Burton, 2010; Payne and Look, 2009; Amatruda and Patton, 2008). Developmental processes can conveniently be tracked using live imaging in zebrafish transgenic for fluorescent proteins, as embryos are transparent and develop externally. Due to their small size and availability in high numbers, zebrafish embryos and larvae are ideally suited for genetic and pharmacological screens (Haffter and Nüsslein-Volhard, 1995; Zon and Peterson, 2005), including screens for altered myelination (Will Talbots screen paper, Bruce Appel paper on myelination mutants, Buckley et al. 2010a). Moreover, in contrast to mammals, adult zebrafish show a remarkably high capacity to regenerate neurons, for example in the retina (Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007) and the spinal cord (Reimer et al., 2008). Severed axons, such as those in the optic projection (Becker et al., 2000) and the spinal cord (Becker et al. 1997), also regrow, ultimately leading to functional recovery (Becker et al., 2004).

Several myelin genes have been characterized in zebrafish. Myelin protein zero (P0) is a structural myelin protein found in compact myelin in the CNS of

zebrafish (Bai et al., 2010; Brösamle and Halpern, 2002; Schweitzer et al., 2003), rather than exclusively in the PNS as in mammals. Gene expression corresponding to that of proteolipid protein (PLP/DM20) has been detected in the CNS only (Brösamle and Halpern, 2002; Yoshida and Macklin, 2005; Schweitzer et al., 2006), while myelin basic protein (MBP), a protein essential for the initiation of myelin compaction, is found in both oligodendrocytes and Schwann cells (Brösamle and Halpern, 2002). The Zwilling –A and –B proteins are also expressed in both, CNS and PNS and have been hypothesized to play roles in the adhesion of myelin lamellae in the zebrafish nervous system (Schaefer and Brösamle, 2009). Contactin (Cntn1/F3/F11), which plays a role in glial cell differentiation and myelination, is expressed in CNS and PNS of developing zebrafish, but was found to be restricted to the uncompact “loose” myelin sheets of the optic axon layer in the retina in adult zebrafish (Schweitzer et al., 2007).

After lesions of the optic nerve or spinal cord, increased expression of L1-related proteins (Bernhardt et al., 1996), *contactin 1a* (Schweitzer et al., 2007), *contactin 1b* (Haenisch et al., 2005), PLP/DM20 (Schweitzer et al., 2006) and P0 (Schweitzer et al., 2003) has been observed in oligodendrocytes that presumably myelinate regenerated axons. Similarly, in goldfish, myelination of regenerated optic axons has been reported (Ankerhold and Stuermer, 1999).

Claudins, which belong to the Peripheral Myelin Protein 22 (PMP22) family, are tetra span trans-membrane proteins that are components of tight junctions in many tissues (reviewed in Findley and Koval, 2009; Loh, Christoffels, Brenner et al., 2004). Tight junctions in myelinating glia, also called autotypic tight junctions as they connect adjacent myelin membranes of the same cell, can be found along the inner and outer mesaxon as well as bordering the edges of Schmidt-Lanterman incisures and paranodal loops (Poliak et al., 2002). To date, 24 proteins of the claudin family

have been identified in humans (Lal-Nag and Morin, 2009) and more than double that number of genes coding for Claudin proteins in teleosts (Loh, Christoffels et al., 2004). However, myelin-specific expression of any particular *claudin* gene has not been demonstrated in fish.

Here we present *claudin k* (also known as *claudin 31*), as a novel myelin-associated gene in zebrafish and describe our anti-Claudin k antibodies and *claudin k:GFP* reporter line as valuable tools for studies of myelin formation as well as myelination of regenerated axons in the adult. Using these tools, we further examine the gene expression profile and morphology of the unique intraretinal oligodendrocytes that form loose wraps of myelin in fish and follow the differentiation of oligodendrocytes in the regenerating optic nerve.

## MATERIAL AND METHODS:

All animal experiments have been approved by the British Home Office or the Bezirksregierung Oberbayern, Germany.

### Fish rising and husbandry

Wild type zebrafish from the Oregon AB and WIK lines and all transgenic lines were raised under controlled conditions at 28.5°C in our fish facility using standard protocols (Westerfield, 1995). Embryos and larvae were obtained from natural matings, raised in embryo medium (15 mM NaCl, 0.5 mM KCl, 1mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 0.15 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05 mM NH<sub>2</sub>PO<sub>4</sub>, 0.7 mM NaHCO<sub>3</sub>) or conditioned aquarium water with 0.00001% methylene blue. Some embryos were raised in the presence of 0.003% n-phenylthiourea from 20 hpf to prevent pigmentation. The following transgenic zebrafish lines were used: *Tg(olig2:egfp)* (Shin et al. 2003), *tg(olig2:Ds-red)* (Kucenas et al., 2008) and the *sox10<sup>l3</sup>* (*colorless*) mutant (Dutton et al. 2001).

### Anti-Claudin k antibody

The C-terminal peptide SPRRAGVSSQVKG<sup>YV</sup> was used for the generation of anti-Claudin k antibodies. The peptide was injected into rabbits to give rise to an immunopositive polyclonal serum. The same peptide was subsequently used to immunize rats and raise the anti-Claudin k rat monoclonal antibody. The rat monoclonal antibody was used in this study unless otherwise stated.

### Plasmid construction

We searched the regions upstream and downstream of the first exon of the *claudin k* gene in different teleosts for phylogenetic conservation by alignment of corresponding genomic sequences from Medaka, Stickleback, and Tetraodon using MultiPIPMaker software. Besides the conserved *claudin k* open reading frame (ORF), two highly conserved areas of approximately 200 bp in close proximity to the first exon, one in the 5' untranslated region and the other in the first intron were identified. These conserved areas are not part of any known transposon or other repeats frequently found in fish genomes (Wasserman and Sandelin, 2004; Das and Dai, 2007), and therefore likely reflect functional parts of the *claudin k* promoter. To include as many regulatory elements as possible, we cloned a 4192 bp long region, starting at the 3' end of the next upstream gene up to, but not including the ORF of *claudin k*, which is located in its entirety in the second exon, and used this sequence to drive expression of the yeast transcription vector Gal4 in a driver vector. A responder vector contained the UAS recognition sequence for Gal4, driving expression of a membrane bound GFP (Paquet et al., 2009). Sequences were flanked by Tol2 sites for efficient integration into the host genome (Kawakami, 2005).

### Zebrafish egg microinjections

Zebrafish eggs were injected with glass-capillaries using standard methods (Holley et al., 2002). The injection solution consisted of *Driver*- and *Responder*-vector (Fig. 7A) and *Tol2*-mRNA at 25ng/μl in DEPC treated dH<sub>2</sub>O containing 20% DEPC treated phenol-red and 0.2M KCL (Kawakami, 2005). Zebrafish embryos were injected into the first cell with ~1 nl injection solution. Injected fish were crossed to wild-type fish and the first filial generation was then screened for fluorescent larvae, some of which were selected to raise stable transgenic lines.



### Antisense Morpholino oligonucleotide injections

Antisense Morpholino (MO) oligonucleotides were designed against the *claudin k* translation start site according to manufacturer's instructions (Gene Tools, LLC, Eugene) (CATGGTGAATCTTTCCCAAGAATGA). As control we used a Morpholino showing no effects in previous studies (Brösamle and Halpern, 2009) (CTGCAGGATTAACGTCTTGATCTTT). All Morpholinos were resuspended in Danieau (58 mM NaCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM CaNO<sub>3</sub>, 5.0 mM HEPES, pH 7.6) and 0.5-1.5 nl of 1 mM MO were injected into the yolk of one- to two-cell stage embryos.

### Immunohistochemistry

Antibodies used were polyclonal rabbit anti-P0 (1:1000, Bai et al., 2011), polyclonal rabbit anti-MBP (1:50, Sigma Genosys), monoclonal LINC (supernatant 1:5, Developmental Studies Hybridoma Bank, IA, USA), monoclonal mouse anti-acetylated tubulin (1:1000, T6793, Sigma Aldrich), mouse anti-ZO1 (ZO1-1A12, 1:20, Invitrogen), polyclonal rabbit and monoclonal rat anti-Claudin k (1:1000, see above). Primary antibodies were detected with appropriate secondary antibodies conjugated to Cy2, Cy3, Cy5, DyLight 488 or DyLight 649 (Strattech Scientific Ltd, England, UK).

*Larval and adult whole mounts:* Zebrafish larvae or adults were terminally anaesthetized by immersion in aminobenzoic acid ethylmethylester (MS222 1:1000 in PBS, Sigma Aldrich). Larvae were washed in PBS, followed by immersion in 4% paraformaldehyde (PFA) and 1% DMSO in PBS for 45 min at room temperature and washed again in PBS. Adults were perfused with 4% PFA, kept in 4% PFA overnight

and the optic nerve and posterior lateral line nerve (PLLN) were dissected out and kept in PBS at 4°C. Just prior to the staining procedure, the larvae and adult tissue were digested with 2 mg/ml collagenase for 20 min or 5 min, respectively, and washed in PBS. The tissue was blocked in 1% normal goat serum, 1% BSA, 1% DMSO, 0.7% TritonX for 30 min followed by incubation with the primary antibody in blocking buffer at 4°C overnight. After washing in PBS, specimens were incubated with the fluorescently labeled secondary antibody in blocking buffer at 4°C overnight. Finally, specimens were washed again and mounted onto slides in 70% glycerol in PBS.

*Teased spinal cord fibers:* Adult fish were killed by immersion in ice cold water (4°C). The spinal cord was exposed to 4% PFA overnight at 4°C, washed in PBS, dissected and teased with fine forceps. Specimens were blocked for 1 hr in 2% normal goat serum / 2% BSA in PBS, followed by incubation with the appropriate primary antibody at 4°C overnight. Specimens were washed extensively in 0.1% Tween20 in PBS, followed by 3 hr incubation in secondary antibody. After the staining procedure fibers were mounted on cover slips with Mowiol (24 g glycerol, 9.6 g Mowiol 4-88, 24 ml dH<sub>2</sub>O, 48 ml 0.2 M Tris, pH8.5).

*Vibrating blade microtome sections:* Adult fish were anaesthetized and fixed as above. Dissected brain tissue was then embedded in 4% agar in PBS and cut into 50 µm slices on a vibrating blade microtome (Microm HM650V). Sections were collected in 24-well plates, washed in PBS, heated in 10 mM sodium citrate in PBS at 80°C for 30 min for antigen retrieval and washed in PBS again. After a 10 min incubation in 50 mM glycine in PBS containing 0.1% TritonX in PBS (PBStx) at room temperature, sections were washed in 0.1% PBStx. Sections were blocked in 2%

normal goat serum (NGS) for 30 min at room temperature followed by an incubation with the primary antibody at 4°C overnight. After washing in 0.1% PBStx, the sections were incubated with the secondary antibody for 45 min at room temperature. The sections were then washed again and mounted as above.

*Cryostat sections:* Zebrafish larvae or adults were terminally anaesthetized as above, and then fixed by immersion in 4% PFA (larvae) or perfusion with 4% PFA (adult). The larvae or dissected tissue from the adults were incubated in 30% sucrose in PBS overnight at 4°C before being embedded in O.C.T. cryostat embedding medium (Tissue Tek, Agar Scientific) and flash frozen in liquid nitrogen. Specimens were then cut into 14 µm sections on a cryostat (Leica CM3050 S) and mounted on Superfrost coated glass slides (VWR International). Sections of larvae and adult tissues were washed in PBS and blocked in 5% NGS in 0.2% PBStx in a wet chamber at room temperature for 1 hr and then incubated in primary antibody in 0.2% PBStx at 4°C overnight. They were washed in PBS, incubated in secondary antibody in 0.2% PBStx for 2 hrs at room temperature and washed in PBS again before being mounted as above.

For retinal sections of adult claudin k:GFP transgenic fish the following modifications of the protocol were used: Fish were terminally anaesthetized as above. The unfixed tissue was then dissected, frozen and cut as above. The sections were treated in ice cold methanol for 10 min, then blocked in 1.5% NGS in PBS for 30 min and then incubated in primary antibody in PBS at 4°C overnight. They were then washed in PBS, incubated in secondary antibody in PBS for 45 min at room temperature and washed in PBS again before being mounted as above.

For retinal sections of *olig2:GFP* transgenic fish the following protocol was used: adult zebrafish were terminally anaesthetized and then perfused. Tissue was

dissected, incubated in sucrose, embedded, frozen and cut as above. The sections were fixed in ice cold ethanol for 10 min, washed in PBS and heated in sodium citrate (pH 6.5) for 10 min at 80°C. Sections were then incubated in blocking solution (10% NGS in 0.3% PBStx) for 1 hr and then in primary antibody in blocking solution at 4°C overnight. They were then washed in PBS, incubated in secondary antibody in blocking solution for 90 min at room temperature and washed in PBS again before being mounted as above.

#### In situ hybridization

To generate the Claudin k probe, EST B1980908 was used with SP6 primers. The probe to detect P0 mRNA has been described previously (Schweitzer et al., 2003). We used standard methods (Thisse and Thisse et al., 1993) to label mRNA in whole zebrafish embryos. In situ hybridization in adult specimens was performed as previously described by Lieberoth et al. (2003). Subsequent immunohistochemistry was performed as above.

#### Western-Blot analysis

Adult zebrafish were terminally anaesthetized as above. Following dissection, tissue was collected in ice-cold lysis buffer containing 20 mM Tris pH7, 150 mM NaCl, 5 mM EGTA, 1% Triton, 0.1% SDS, Protease inhibitor 1:100. The homogenized tissue was then incubated on ice for 30 min. After centrifugation at 6000rpm for 5min, the supernatant was taken for protein quantification (Micro BCA Protein Assay Kit - PIERCE Biotechnology, Rockford, IL, USA). The homogenized tissue samples were diluted with loading buffer (NuPAGE 4X SDS Sample buffer, Invitrogen, Paisley, UK) and boiled at 95°C for 5min. After loading the samples onto Precise™ protein gels 4-20% (Thermo Scientific, UK), they were run at 100 V for 1-

1.5 hrs in HEPES running buffer (Thermo Scientific, UK). The bands were then transferred onto a PVDF membrane (Millipore, UK), which was fixed in methanol and distilled water for 15 seconds prior to the transfer, at 400 mA for 2 hrs. Following the transfer, the membrane was blocked in blocking buffer (1X TBS with 0.1% Tween20 and 4% BSA) for 1 hr at rtp and incubated with primary antibody in blocking buffer overnight. The membrane was washed in TBST (1X TBS with 0.1% Tween20), incubated with HRP-labeled secondary antibody (GE healthcare Ltd, UK) in blocking buffer at room temperature for 1 hour and washed in TBST again. They were then developed in developing spray (Calbiochem, Nottingham, UK), and exposed to film in the dark room for 0.5-4 min.

### Live imaging

Zebrafish larvae were anaesthetized by immersion in aminobenzoic acid ethylmethylester (MS222 1:5000 in PBS, Sigma Adrich, Dorset, UK) and embedded in 1.5% agarose (RESolve™ Low Melt Agarose, Geneflow, Fradley, UK) in PBS. The larvae were placed and orientated on the coverslip in a drop of agarose. Finally, they were mounted on a glass slide in aminobenzoic acid ethylmethylester and embryo medium and imaged under a Zeiss LSM 710 confocal microscope. After imaging, the larva was taken out of the agarose and placed in fresh embryo medium for further growth and development. This imaging process was repeated with the same larva at intervals of 12 hours.

### Optic nerve crush

Adult zebrafish were anesthetized by immersion in aminobenzoic acid ethylmethylester (MS222 1:5000 in PBS, Sigma Aldrich, Dorset, UK) and transferred to a wet surface on ice. Under visual control (stereomicroscope), connective tissue

surrounding the eye was pulled away with forceps. The eye was then gently lifted out of the eye socket to expose the optic nerve, which was compressed using Dumont (Hatfield, PA, USA) No. 5 forceps. A clear stripe over the otherwise opaque nerve indicated a successful lesion. The eye was rotated back into the eye socket and the fish was returned into a tank.

#### Quantification of myelination

Confocal images were taken and converted into stacks as described below. Using Image J (Version 1.44p), pixel intensities were measured using the same size square in the lesion site area of the optic nerve and compared to the non-lesioned site. Statistical analysis was performed using a One-Way ANOVA with a Bonferroni post-test using the Prism 5 software (Graphpad).

#### Microscopy, image acquisition and processing

Confocal images were taken on a Zeiss LSM 710 confocal microscope using the Zeiss ZEN 2009 software. Confocal images of crushed optic nerves were taken on a Leica DMI4000B confocal microscope using the Leica Application Suite AF software. Images of immunofluorescence and in situ hybridizations were taken on a Zeiss Axio Scope A1 using the Zeiss Axio Scope A1 software. All images were extracted and stacked in Image J and processed using Adobe Photoshop CS4. Image contrast was adjusted by using the “curves-adjustment”-tool in Photoshop.

## RESULTS:

### Cloning of Claudin k in zebrafish

The *claudin k* gene maps to chromosome 3 in zebrafish at 48,270,951-48,274,629 bp, and consists of 2 exons (Fig. 1A). It was originally named *claudin 31*, based on its sequence homology to *Fugu claudin 31* (Loh et al., 2004), and later renamed claudin K to satisfy nomenclature criteria (ZFIN.org). The entire 648 bp ORF lies on the second exon and contains two synonymous coding single nucleotide polymorphisms (SNP) and one non-synonymous SNP. The Claudin k protein is 216 aa long, and has a calculated molecular weight of 22.8 kDa. Computational analysis by TMHMM 2.0 predicts the typical topology of Claudins (Fig. 1B), with 4 transmembrane domains, a short intracellular N-terminus, a longer C-terminus and a long, first extracellular loop. The length of its N- and C-terminus and its intracellular and extracellular loops, are consistent with other classical Claudins. Conserved putative domains and functional sites were identified with Prosearch, PPSearch and according to Krause et al. (2008). By phylogenetic analysis, *claudin k* clusters with other *claudins* (Fig. 1C). The orthologues *Takifugu rubripes claudin 31* (81% aa identity) (Loh et al., 2004) and *Salmo salar claudin 6* (82% aa identity, Leong et al., 2010) show a high sequence homology, whereas the mammalian *claudin* with the highest sequence homology is mouse *claudin 6* (61% aa identity) (Diez-Roux et al., 2011).

### Developmental expression of *claudin k* mRNA

We examined the mRNA expression pattern of *claudin k* at different developmental stages using in situ hybridization. In whole mounted zebrafish larvae, expression was observed at 2 days post fertilization (dpf) in the hindbrain and rostral

spinal cord (Fig 2. A-D), the location in which the first oligodendrocytes differentiate (Brösamle and Halpern, 2002). At 3 dpf, *claudin k* expression in the hindbrain and spinal cord was more extensive and also detectable in cells of the dorsal longitudinal fascicle, in presumed Schwann cells of the posterior lateral line (PLL) nerve and along the nerves innervating the extraorbital eye muscles (Fig 2. E-K). A transient expression domain in the position of the epiphysis in the rostral part of the zebrafish head was also detected at 2 dpf, becoming weaker at 3 dpf (Fig. 2A,E). Thus early expression patterns of *claudin k* mRNA are consistent with expression in oligodendrocytes and Schwann cells.

#### Development of specific anti-Claudin k antibodies

To characterize Claudin k protein expression, we generated specific antibodies using a peptide from the C-terminus (SPRRAGVSSQVKG YV), as this is the most divergent part of the Claudin proteins. We raised an anti-Claudin k rabbit polyclonal and a rat monoclonal antibody. Both the monoclonal and the polyclonal antibody recognized a single band at the expected size of ~20 kDa on Western blots using either peripheral or central nervous system tissue of adult zebrafish (Fig. 3A). In whole mounted zebrafish larvae at 5 dpf, the Claudin k antibody label structures that correspond to myelinated structures in the CNS and PNS, such as the Mauthner axons and the PLL nerve (Fig. 3B). Both monoclonal and polyclonal antibodies show identical patterns of staining (data not shown). Using anti-sense Morpholino knock down, Claudin k immunoreactivity was abolished (Fig. 3C), indicating specificity of the antibody for Claudin k. Claudin k immunoreactivity, along with mRNA expression and other myelin markers, was reduced in whole-mounts and cryostat sections of the hypomyelinating *sox10<sup>l3</sup>* (*colorless*) mutant, supporting myelin-associated labeling



(Fig. 4). Thus, we generated antibodies that specifically recognize Claudin k in zebrafish.

#### Claudin k protein localises to tight junctions of myelinating cells

To determine the subcellular localisation of Claudin k, we used the anti-Claudin k antibody on adult tissue. In cross sections of a peripheral nerve, anti-Claudin k immuno-labeling was observed surrounding axons in the PNS (Fig. 5A). The characteristic large diameter Mauthner axon in spinal cord cross sections was also surrounded by Claudin k immunoreactivity (Fig. 5B). In teased fibers of the spinal cord and peripheral nerve, Claudin k localisation was found in the innermost mesaxon, Schmidt-Lanterman incisures and the paranodes of Schwann cells and oligodendrocytes (Fig. 5C,D). As Claudins are typically located in tight junctions, which are present in these locations, we double-labeled adult peripheral nerve fibers with the Claudin k antibody and an antibody to the zona occludens-1 protein (ZO-1), which is associated with tight junctions (Poliak et al., 2002), and found that ZO-1 labeling co-localised with that of the Claudin k (Fig. 5E). These observations suggest that Claudin k is a tight junction protein in oligodendrocyte and Schwann cell myelin.

#### Generation of *claudin k*:GFP transgenic zebrafish

Claudin k immunoreactivity can only be demonstrated in fixed material and does not label the entire morphology of the myelinating cells. To overcome these limitations, we generated a transgenic zebrafish, in which the *claudin k* promoter drives expression of a membrane-bound green fluorescent protein (GFP). We used the previously described UAS-Gal4 system to achieve high transgene expression (Fig. 6A). Overall, transgene expression corresponded to Claudin k immunoreactivity (see below). To further determine whether transgene expression occurred in

oligodendrocytes, we crossed this fish with an already characterized line, *olig2:DsRed*, in which cytoplasmic DsRed is expressed under the control of the *olig2* promoter in oligodendrocytes and specific neurons. In the spinal cord, dorsally migrating *olig2:DsRed*<sup>+</sup> cells have been identified as oligodendrocytes (Kucenas et al., 2008). Some of these were surrounded by a Claudin k-labeled GFP-positive membrane, which continued into processes that ended in longitudinal structures reminiscent of myelinated internodes (Fig. 6B,C). Gaps in the labeling are suggestive of nodes of Ranvier and were found in the CNS and PNS (Fig. 6B,D). Immunolabeling for Claudin k in whole mounted larvae showed that positive staining for the Claudin k protein and GFP was localised to the same myelin sheaths (Fig. 6C). Of note, the transgenically expressed and membrane-localized protein labeled the oligodendrocyte or Schwann cell body and myelin sheaths, whereas the antibody labeling was only found in the myelin. Thus the *claudin k*:GFP transgene reports the morphology of *claudin k* expressing myelinating cells.

#### Developmental expression of Claudin k and *claudin k*:GFP.

Next we analyzed protein and transgene expression of *claudin k* in cryostat sections of developing zebrafish at 1, 2, 3, 4 and 5 dpf and compared these patterns to those of other myelin markers. Claudin k immunoreactivity was first detectable at 3 dpf in the hindbrain (Fig. 7A), spinal cord and posterior lateral line nerve (data not shown). At 4 dpf expression was first detectable in the optic chiasm (Fig. 7B). Over time, myelination increased in these locations and throughout the CNS and PNS.

*Claudin k*:GFP was already present at 24 hpf in cells in the rostral head (data not shown), reflecting early *claudin k* mRNA expression (Fig. 2), but these labeled cells are unlikely to be oligodendrocytes at this early developmental stage. The onset of GFP expression in presumed myelinating cells in the hindbrain was detected at 2

dpf (Fig. 7A). Labeling was also found in the optic chiasm (Fig. 7B), spinal cord and posterior lateral line nerve at this time point (data not shown). Thus, transgene labeling first appeared in the same structures as Claudin k immunoreactivity one to two days earlier than the immunoreactivity. Thereafter the signal expanded over the entire CNS and PNS.

MBP and P0 immunoreactivity in chiasm, hindbrain, spinal cord and PLL could only be detected from 4 and 5 dpf, respectively (Fig. 7A,B). In summary, Claudin k and the transgene can be detected in all areas that have previously been shown to express myelination markers (Brösamle and Halpern, 2002). P0 and MBP immunoreactivities confirm these locations. Claudin K antibodies and transgenic fish are therefore excellent tools for the visualization of myelination.

Next, we tested whether the *claudin k*:GFP transgene could be used to analyze myelin formation in individual live larvae. In lateral (Fig. 8) and dorsal (Fig. 9) views, first expression was detected at 54hpf in the medial longitudinal fascicle in the hindbrain, structures medial of the otic cavity, as well as in the rostral spinal cord and PLL (Figs. 8A, 9A). No signal was detectable in GFP-negative control larvae (Figs. 8B, 9B). As individual larvae developed, GFP expression extended along the spinal cord and PLL in a rostral to caudal pattern (Fig. 8A), similar to previously published myelination patterns (Brösamle and Halpern, 2002). Motor axons, which were visible in red fluorescence, using the double transgenic fish *olig2*:DsRed/*claudin k*:GFP described above, and the dorsal longitudinal fascicle of the spinal cord showed myelination, determined by *claudin k*: GFP fluorescence, from 78 hpf onwards (Fig. 7C). GFP expression was also detected in the fins of the zebrafish larvae (Fig. 8A), but this expression could not be co-labeled with anti-Claudin k antibody (data not shown), and therefore was deemed ectopic. However this expression did not affect

observation of myelin in the nervous system. Thus the progress of myelination can be followed in live *claudin k*:GFP transgenic animals.

#### Expression of Claudin k and *claudin k*:GFP in adult zebrafish

In adult zebrafish, Claudin k and transgenic GFP expression were detectable throughout the CNS, e.g. in the telencephalon (Fig. 10C), tectum and tegmentum (Fig. 10A), labeling myelinated structures, such as the tectobulbar tract (Claudin k antibody and *claudin k*:GFP), as well as oligodendroglial cell bodies (*claudin k*:GFP only). In the PNS, as exemplified by the PLL (Fig. 10D), immunofluorescence and transgene expression was also detected and peripheral nerves surrounding the eye can be seen through the skin in live fish (Fig. 10E). Double labeling with P0 and MBP antibodies (data not shown) confirmed the myelin-specific labeling pattern of the antibody, whereas double-labeling of GFP+ cell bodies with P0 mRNA, confirmed labeling of oligodendrocyte cell bodies by GFP (Fig. 10B).

However, GFP did not label all myelinated fibers that can be detected with anti-Claudin k or anti-P0 immuno-labeling (Fig. 10C). To quantify this, we counted GFP labeled cells in vibrating blade microtome sections of the outer layers of the tectum in *olig2*:GFP and *claudin k*:GFP transgenic fish. We found that *claudin k*:GFP only labeled approximately half the number of glial cells compared to the *olig2*:GFP fish (*claudin k*:GFP:  $16.3 \pm 3.3$  cells per  $0.002\text{mm}^3$ , *olig2*:GFP:  $37.2 \pm 6.8$  cells per  $0.002\text{mm}^3$ ,  $n=3$ ,  $p \leq 0.01$ , one-tailed t-test). As we know that *olig2* is also present in cerebellar neurons (McFarland et al., 2008) and might therefore not be an exclusive oligodendrocyte marker in the tectum, we also investigated oligodendrocyte specific P0 mRNA and *claudin k*:GFP labeling and observed similar differences in labeling of P0 mRNA and GFP signal (data not shown). Thus Claudin k immunoreactivity

quantitatively labels myelin, whereas *claudin k*:GFP exhibits a mosaic expression pattern in the adult CNS.

### Myelination in the optic system of adult zebrafish

Retinal ganglion cell (RGC) axons show distinct myelination patterns along their length depending on the domain of the axon and regenerate after a crush lesion, prompting detailed analysis of Claudin k expression in this system. The intra-retinal part of the axons is wrapped in glial membranes termed “loose myelin”. Of note, *contactin 1a* mRNA expression, usually only found in actively myelinating cells, is retained in glial cells of the RGC axon layer in the retina, whereas the marker of compact myelin, P0, is not found in the retina (Schweitzer et al., 2003). Outside the retina, in the optic nerve and tract, strong P0 expression but no *contactin 1a* expression is found (Schweitzer et al., 2007). Using the Claudin k antibody and transgenic *claudin k*:GFP line, we find that Claudin k is expressed in the retina and optic nerve (Fig. 11A). MBP immunoreactivity shows a pattern similar to Claudin k immunoreactivity (Fig. 11B). By comparison, the P0 antibody only labels the optic nerve and immunoreactivity cannot be detected in the retina (Fig 11B), confirming earlier *in situ* hybridization results. In the *olig2*:GFP line, intra-retinal cells in the optic axon layer, as well as cells in the optic nerve were also labeled, confirming that oligodendroglial cells are present in both locations (Fig. 11C).

We used the mosaic labeling in *claudin k*:GFP fish to our advantage to visualize single oligodendrocyte cell bodies in the retina (Fig. 11D) and optic nerve (Fig. 11E). We found labeled cells of typical oligodendrocyte morphology with multiple processes and myelin sheaths (Grinspan, 2002) both in the retina and the optic nerve. To our knowledge, this is the first time that the full morphology of retinal

oligodendrocytes has been detected. Together with the presence of *olig2:GFP* and *contactin 1a* expression in the optic axon layer of the retina, these observations support the presence of oligodendrocytes inside the retina, producing loose myelin around intra-retinal axons. In contrast, oligodendrocytes in the optic nerve produce compact myelin.

### Myelin dynamics in the lesioned adult optic nerve

The optic system of the zebrafish is an easily accessible system to study nerve regeneration and related changes in myelination. Previous work has shown that axons damaged by an optic nerve crush degenerate within hours and fully regrow from the retinal (proximal end) to fully reinnervate the tectum (distal) by 4 weeks post-lesion (Becker and Becker, 2000), with most axons having repopulated the entire optic nerve already by 16 dpl (Wyatt et al., 2010). We investigated the dynamics of Claudin k and *claudin k:GFP* transgene expression as markers of myelin formation and LINC immunoreactivity, as marker of axonal regeneration, after optic nerve crush in adult zebrafish (Fig. 12A,B). To quantify changes in immunoreactivity, we measured the intensity of immunofluorescence in the lesion site and described this as a ratio to unlesioned control staining intensity (Fig. 12C).

At 3 days post-lesion (dpl) (the earliest time point analyzed) in *claudin k:GFP* zebrafish, we observed significantly reduced Claudin k and LINC immunoreactivity and GFP labeling in the lesion site, indicating a loss of myelinating cells and axons. Proximal and distal to the lesion site, immunofluorescence persisted due to intact axonal segments (proximal) and fiber debris (distal), and prevented us from quantifying changes in these regions. At 7 dpl Claudin k immunoreactivity and GFP labeling was still reduced, however GFP-positive cell bodies were observed in the

lesion site. Anti-LINC labeling had increased from 3 dpl levels, suggesting axonal regrowth had started at this time point. At 14 dpl, we observed GFP+ oligodendroglial cells in the lesion site, some with only a limited number of processes and some with more complex morphologies, suggestive of immature and more differentiated oligodendroglial cells, respectively (Fig. 13A). At 28 dpl, Claudin k and LINC immunoreactivities, as well as *claudin k*:GFP fluorescence had returned to levels that were similar to those in the unlesioned control nerve, supporting the interpretation that axons had regenerated and were myelinated again.

Mosaic labeling in the transgenic fish allowed assessment of oligodendroglial morphology and number during the regeneration process. We quantified GFP-positive cells within 200  $\mu\text{m}$  centered around the lesion site (the average extent of the lesion) in longitudinal 14  $\mu\text{m}$  sections over the period described above (Fig. 13B). We observed an overall increase from 4 cells ( $\pm 1.4$ , 3 days post-lesion,  $n=2$ ) to 17.7 ( $\pm 3.5$ , 28 days post-lesion,  $n=3$ ) in the lesion site. We observed that GFP+ cells with a simple morphology ( $\leq 2$  processes) in the lesion site were present at 3 dpl ( $4 \pm 1.4$  cells, ns,  $n=2$ ), significantly increased at 7 dpl ( $15 \pm 1.4$  cells,  $p \leq 0.05$ ,  $n=2$ ) and decreased again at 14 dpl ( $4 \pm 3.6$  cells, ns,  $n=3$ ) and 28 dpl ( $0.3 \pm 0.6$  cells, ns,  $n=3$ ). By comparison, cells with a complex oligodendrocyte-like morphology ( $> 2$  processes) were not found in the lesion site at 3 dpl at all, but increased in number during the regeneration process (7 dpl:  $3.5 \pm 2.1$  cells, ns,  $n=2$ ; 14 dpl:  $14.7 \pm 9.8$  cells, ns,  $n=3$ ; 28 dpl:  $17.3 \pm 3.5$  cells, ns,  $n=3$ ).

## DISCUSSION:

In this study, we describe a novel myelin associated protein, Claudin k, which localises to mesaxon, Schmidt-Lanterman incisures and paranodal loops in the myelin sheath of zebrafish PNS and CNS. We find dynamic expression patterns during development and optic nerve regeneration, in accordance with previously described processes of myelin formation, regeneration and re-myelination (references Brösamle and Halpern, Becker, others?). A *claudin k*:GFP reporter fish allowed the morphological characterization of intra-retinal oligodendrocytes, forming loose myelin, and the transition from simple to complex morphologies of oligodendrocytes during optic nerve regeneration.

### Claudin k expression compared to other myelin markers

All proteins of the Claudin family which have been classified to date are components of tight junctions in various tissues. We find that *claudin k* expression co-localises with the tight junction marker ZO-1 in peripheral and central nerves, suggesting that Claudin k is specifically localised to autotypic tight junctions in myelinating Schwann cells and oligodendrocytes. We show that the first detectable mRNA expression of Claudin k is at 2 dpf in the hindbrain of zebrafish larvae, corresponding to the onset of myelination, and consistent with mRNA detection of other myelin markers such as P0, DM20 (proteolipid protein) and MBP (Brösamle and Halpern, 2002). Similarly, Claudin k protein is first detectable in the medulla and optic chiasm at 3 dpf and 4 dpf, respectively. It is thus one of the earliest markers for myelin found in zebrafish. Reduction in the amount of myelin, by mutation (*sox10* deficient fish) or by optic nerve crush, reduces Claudin k protein expression. After the optic nerve crush, GFP expression in our transgenic *claudin k*:GFP fish is also



reduced. Thus, Claudin k immunoreactivity and the *claudin k*:GFP transgenic fish can be used to track myelination in development and in adult zebrafish.

#### Mosaic expression of the *claudin k*:GFP transgene

In adult zebrafish, we observed a mosaic expression pattern of *claudin k*:GFP, where GFP is not expressed consistently in all myelin sheaths with less labeling of myelin than can be detected by antibody staining. This however, is not the case in *claudin k*:GFP larvae, where we find a homogenous transgene expression pattern. We attempted several in-crossing and out-crossing breeding strategies to make the line stable and maximize the number of integrated transgene copies, however, we continued to observe this mosaic expression pattern. We assume that this might be due either to differential gene regulation or integration in the adult fish or the use of the UAS-GAL4 system used for the generation of the transgenic fish as gene silencing has been found in this system previously (Akitake et al., 2011). However, although the GFP expression only labels a proportion of myelinated fibers in adult fish, it labels the entire morphology of single myelinating cells, which provides a unique opportunity to use our *claudin k*:GFP fish for the study of these cells.

Loose myelin in the zebrafish retina

In our study of myelination in the visual system of zebrafish, we show that Claudin k and MBP are expressed in optic nerve and retina, while P0, a marker of compact myelin, is only detectable in the optic nerve. In previous research we have shown that the zebrafish optic nerve contains cells expressing P0 mRNA (Schweitzer

et al., 2003) and that the optic nerve is surrounded by compact myelin, whereas intra-retinal axons, by comparison, are only ensheathed by loose wraps of cell membrane (Schweitzer et al., 2007). This is consistent with findings in goldfish and other teleosts (Wolburg, 1980; Easter et al., 1984). We show that these wraps of membrane contain myelin proteins and that there are *olig2* expressing glial cells located throughout the retina, which express *olig2*, MBP and Claudin k protein, supporting the notion that they are indeed oligodendrocytes. However, unlike those in the optic nerve, intra-retinal oligodendrocytes appear unable to produce either P0 or compact myelin. Capitalizing on GFP expression in both cell body and myelin sheath in *claudin k:GFP* transgenic fish we demonstrate the morphology of these intra-retinal cells as consistent with oligodendrocytes, with several processes emanating from the cell body to enwrap axons in loose myelin.

Most mammals do not have myelin in the retina, due to the prevention of oligodendroglial precursor? cell migration into the retina (French-Constant et al., 1988) at the lamina cribrosa at the optic nerve head. However, when oligodendrocyte precursors are injected into the retina they are capable of forming compact myelin (Setzu et al., 2004; Setzu et al., 2006), indicating that in mammals intra-retinal axons are fully myelination competent. This raises the intriguing possibility that in fish environmental factors prevent retinal oligodendrocytes from forming compact myelin. However, intrinsic differences between retinal and extra-retinal oligodendrocytes cannot be excluded. In multiple sclerosis, failure of remyelination has been attributed in part to a failure of oligodendroglial cells to fully mature into myelinating oligodendrocytes once they have migrated to the area of damage (reviewed in Piaton et al. 2009). Thus, the zebrafish retina may provide a useful tool for studying factors that prevent oligodendrocytes from forming compact myelin.

### Regeneration in the visual system

We show that at 3 days after optic nerve crush, Claudin k immunoreactivity is severely reduced, and has recovered again between 14-28 days after nerve crush. This corresponds with findings in goldfish optic nerve myelination studies, which describe the reappearance of the myelin molecules 36K protein, galactocerebroside and MBP 3.5 weeks post optic nerve crush (Ankerhold and Stuermer, 1999). At early times after optic nerve crush in the *claudin k*:GFP fish, we identified GFP-positive cells around the crush site showing a simple bipolar morphology, characteristic of oligodendrocyte precursor cells (Grinspan, 2002). However, these cells could also be invading Schwann cells, as has been observed in goldfish (Nona et al., 1992). Nevertheless, at progressively later stages of optic nerve regeneration multipolar myelinating GFP-positive cells had many processes and were surrounded by large numbers of myelinated axons, a clear indication of oligodendroglial identity. It is as yet unknown if these oligodendroglial cells newly differentiate from OPCs or whether mature, damaged oligodendrocytes are capable of de-differentiating into immature oligodendroglial cells and re-differentiating to be able to myelinate regenerated axons. In the mammalian CNS, remyelination occurs only from oligodendrocytes newly differentiating from OPCs, but in the PNS, Schwann cells are capable of de- and re-differentiation. Bearing in mind that some myelin proteins found in zebrafish CNS myelin are only found in mammalian PNS myelin (e.g. P0), it will be interesting to determine how re-establishment of a myelin sheath is achieved in zebrafish.

### Potential function of claudin k

Claudin k localises to mesaxon, Schmidt-Lantermann incisures and paranodal loops in CNS and PNS myelin of zebrafish and co-localises with ZO-1 in peripheral nerves, which is specific to tight junctions. This expression is similar to the CNS and

PNS specific Claudin 11 and 19 in mammalian myelin. Transgenic mice, lacking CNS Claudin 11 or PNS Claudin 19, lack tight junctions, while myelin development, ultrastructure and maintenance appear to be unaffected (Gow et al., 1999; Miyamoto et al., 2005). Electrophysiological examination of these knockout mice revealed nerve conduction deficits, suggesting roles of myelin-specific Claudins, such as Claudin k, in electrical sealing of the myelin sheath.

### Conclusion

We have described and characterized Claudin k in zebrafish, finding it present in tight junctions of myelinating cells, and have shown that it is a reliable marker for myelin corresponding in its expression to other myelin markers. We have developed a useful transgenic line, *claudin k*:GFP, in which membrane bound GFP expressed under the *claudin k* promoter not only labels myelin sheaths, but also oligodendrocyte cell bodies. We believe that this will be useful for screens of small molecules for their effect on oligodendroglial cell number, migration and myelination in development, as well as myelin regeneration studies in adult fish, to investigate the relationship between oligodendrocytes and their myelin sheath. We have performed proof of principle studies showing that loss and subsequent gain of Claudin k protein and a *claudin k*:GFP transgene can be used to follow myelin-related changes during degeneration and regeneration of CNS tracts.

#### AUTHORS' CONTRIBUTIONS:

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## Figures

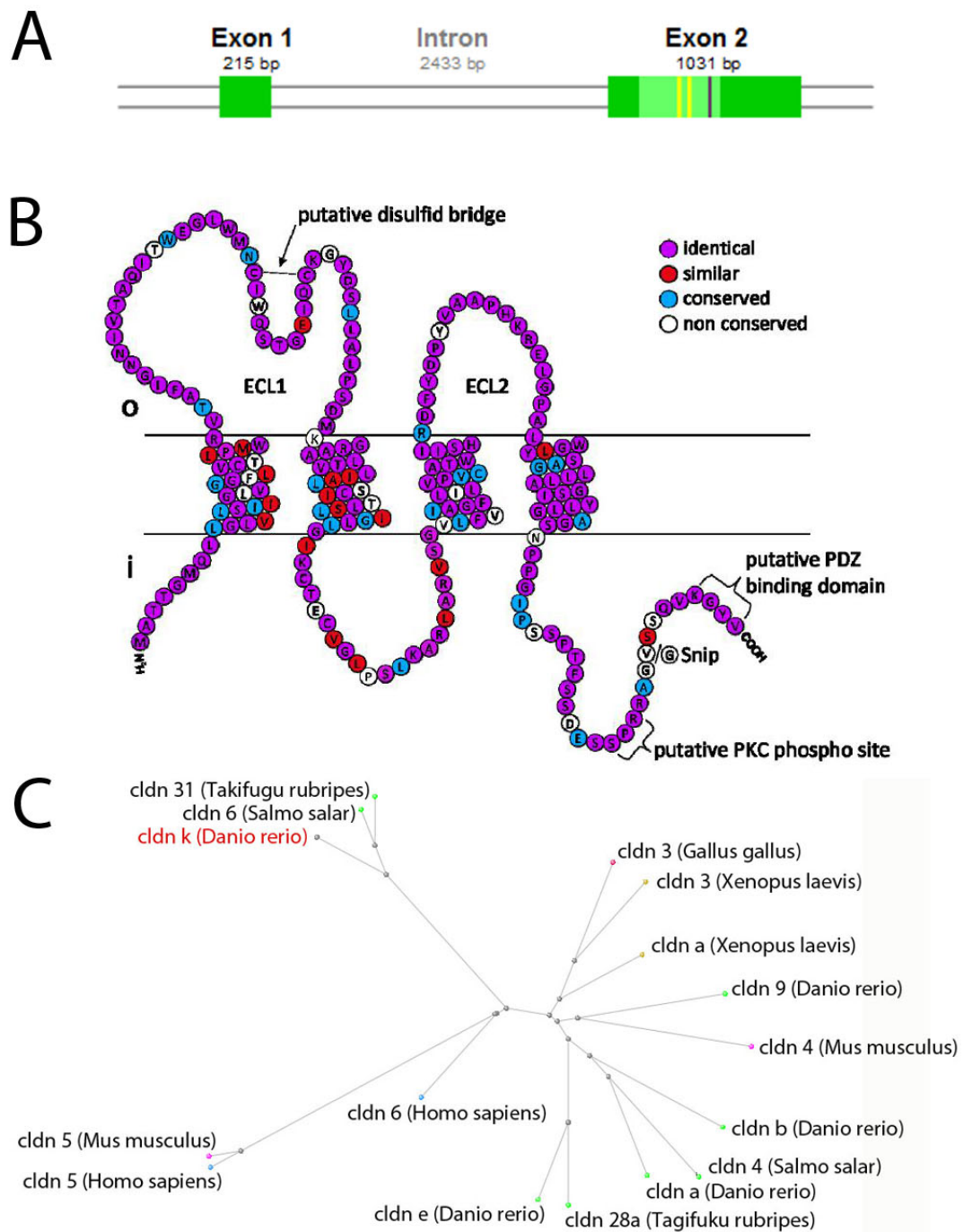


FIGURE 1: The *claudin k* gene. A: *Claudin k* locates to chromosome 3 at 48,270,951-48,274,629 bp on the forward strand and consists of two exons separated by an intron. The whole ORF of 648 bp (light green) lies on the second exon and contains two synonymous coding SNPs (yellow) and one non-synonymous coding SNP



(purple). B: Claudin k protein sequence and predicted membrane topology, according to TMHMM 2.0. The colour-code indicating conserved residues is based on ClustalW alignment of 9 teleost Claudin k and TeXshade 1.4. C: Phylogenetic tree of Claudin k and related Claudins.

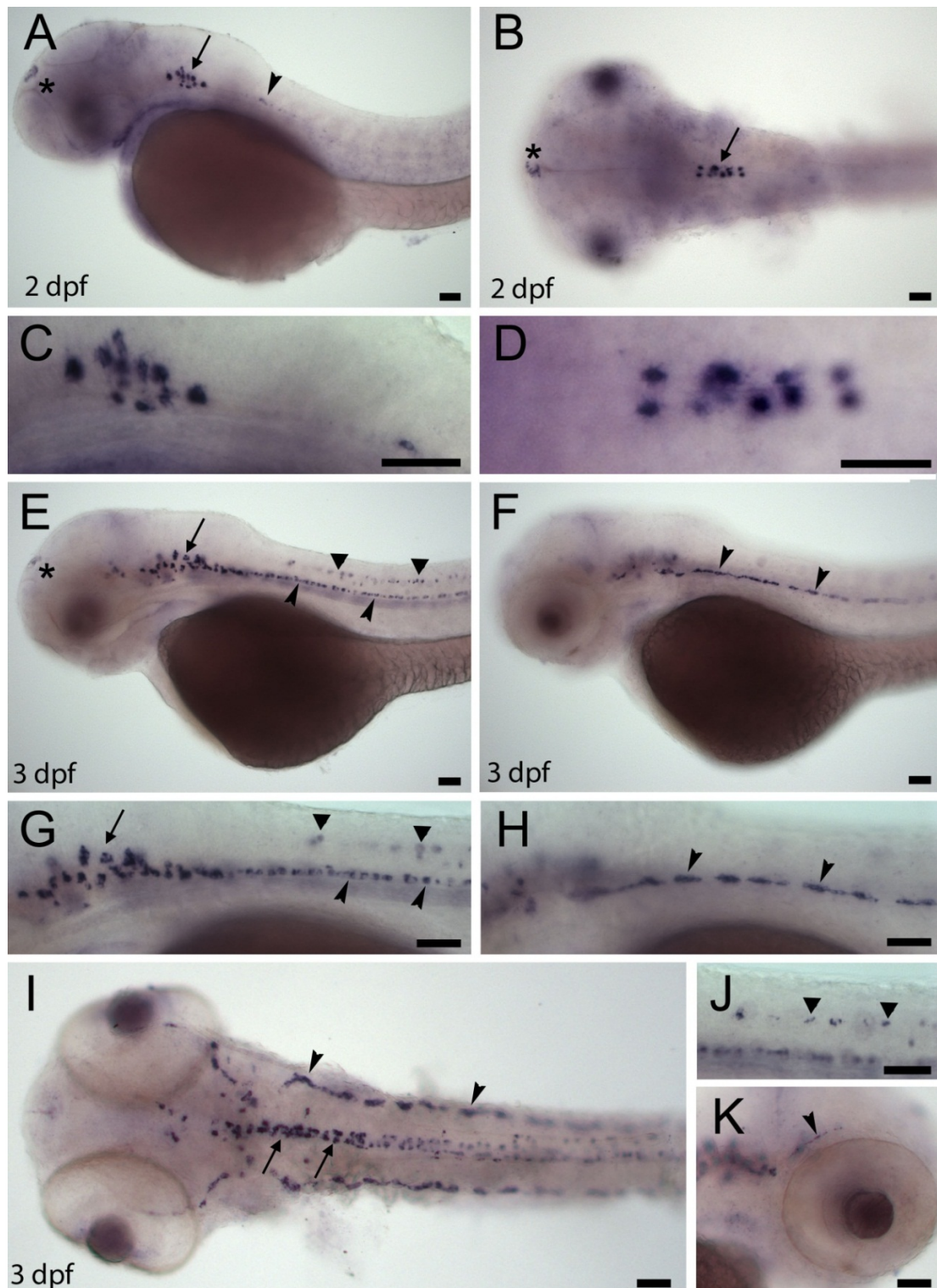


FIGURE 2: Claudin k mRNA expression in whole-mount embryos at 2 and 3 dpf. A-D: At 2 dpf, first expression was detected in oligodendrocytes of the hindbrain (*arrow*) and in the spinal cord (*arrowhead*). Expression in the position of the epiphysis was detected in the rostral part of the zebrafish head (*asterisk*). E and G: At 3 dpf, Claudin

k is strongly expressed in oligodendrocytes of the hindbrain (*arrows*), the ventral reticulospinal tracts (*arrowheads*) and the dorsal longitudinal fascicle (*triangles*). The expression domain in the rostral part of the zebrafish head becomes weaker (asterisk). E: lateral view. G: higher magnification. F and H: Expression can also be found in the Schwann cells of the PLL nerve (*arrowheads*). F: lateral view. H: higher magnification. I: Dorsal view of Claudin k expression at 3 dpf in oligodendrocytes of the spinal cord (*arrows*) and Schwann cells in the PLL (*arrowheads*). K: Schwann cells along the nerve innervating the extraorbital muscles of the eye. Scale bars 50µm.

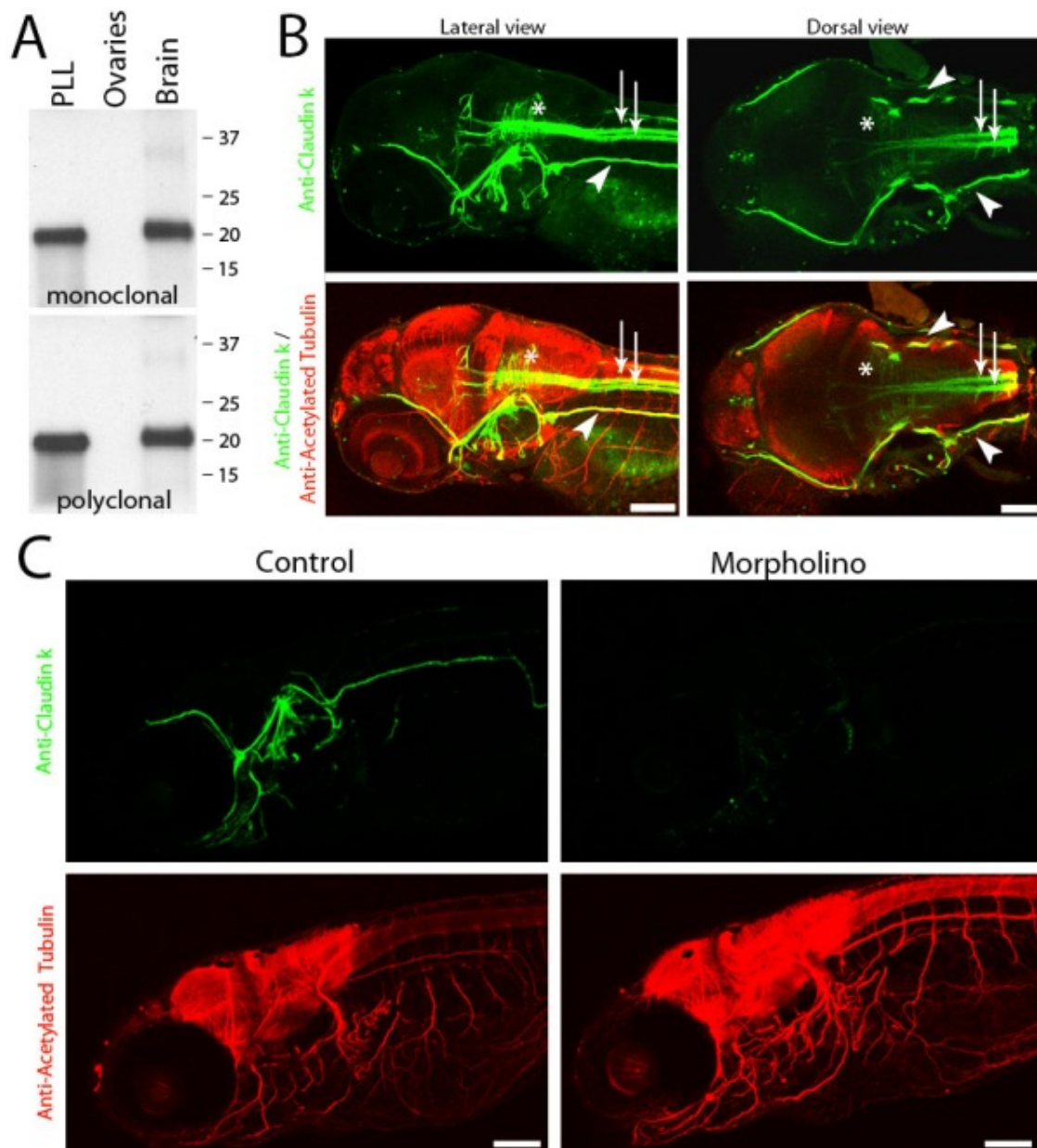


FIGURE 3: Claudin k antibody specifically labels myelinated structures in zebrafish.

A: Specific bands for Claudin k are detected with the monoclonal and polyclonal antibody at ~20kDa in the central and peripheral nervous system (brain and posterior lateral line nerve, respectively). The negative control (ovaries) shows no Claudin k band. B: Dorsal and lateral view of Claudin k antibody staining in whole mounted zebrafish larvae at 5 dpf is shown (rostral is left). Claudin k labeling is shown in green and neuronal processes are labeled with an antibody to acetylated tubulin in red.

Myelin sheaths of the Mauthner axons (*arrows*) are also labeled. In the mid- and hindbrain, very thin processes cross the midline (*asterisk*). The posterior lateral line nerve can also be identified (*arrowheads*). C: Whole mount larvae at 5 dpf (lateral view, rostral is left, dorsal is up) injected with control and Claudin k Morpholino, labeled with Claudin k and anti-acetylated tubulin antibodies, is shown. Claudin k labeling is strongly reduced in Morpholino-injected larvae. Scale bars: 100µm.

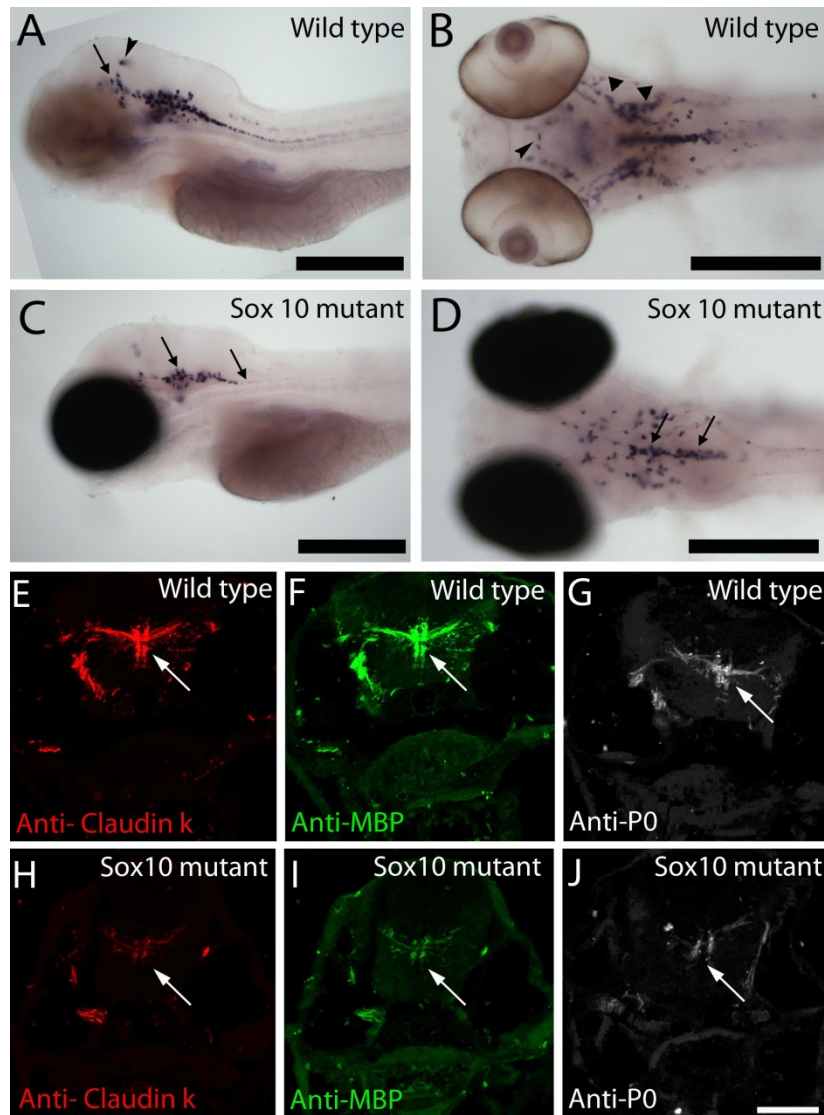


FIGURE 4: Claudin k expression is reduced in *sox 10* mutant larvae. A-D: Lateral (A,C) and dorsal (B,D) views of whole-mounted embryos are shown (rostral is left). At 4 dpf, Claudin k mRNA can be found in structures of the midbrain (A, *arrowhead* and *arrow*) as well as in the optic chiasm (B, *arrowhead*) and around the developing ear (B, *triangles*). Expression of Claudin k mRNA in *sox 10* mutants of the same age is severely reduced in hindbrain and spinal cord (C, D, *arrows*). E-J: Immunolabeling of different myelin markers in cross sections of the hindbrain of 8 dpf zebrafish larvae is shown. Myelination in the medial longitudinal fascicle (*arrows*) is severely reduced in

the *sox 10* mutants compared to wild type fish of the same age. E, F and H, I show double-immunohistochemistry of the same tissue section, respectively. Scale bars: A-D = 200 $\mu$ m, E-J=100 $\mu$ m.



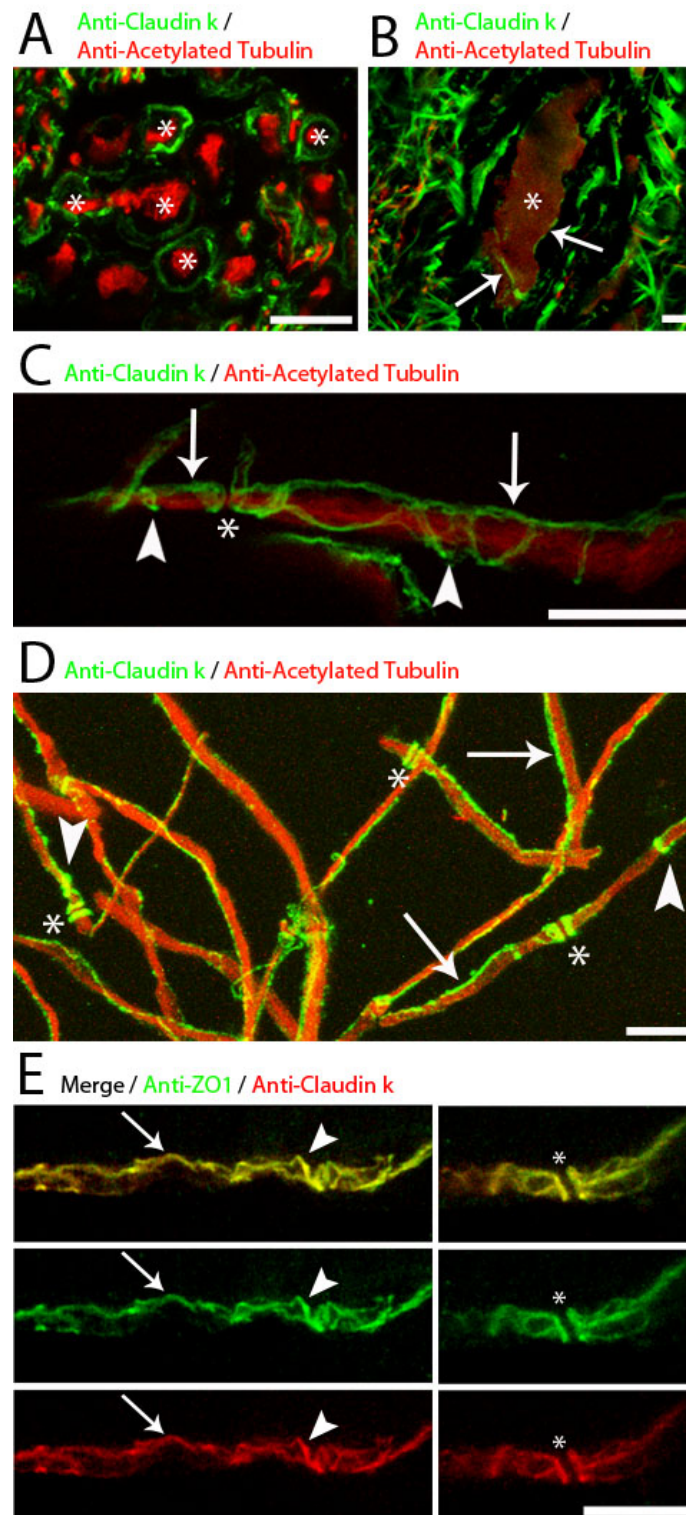


FIGURE 5: Claudin k localises to autotypic tight junctions in adult zebrafish myelin.

A: In a cross section of a peripheral nerve, anti-Claudin k labeled myelin surrounds some axons (*asterisks*). B: In a cross section of the spinal cord, Claudin k

immunolabelling (*arrow*) can be observed around the Mauthner axon (*asterisk*). C: A

longitudinal section of a peripheral nerve, anti-Claudin k labeled myelin surrounds some axons (*asterisks*). D: In a longitudinal section of a peripheral nerve, Claudin k



teased fiber preparation of a single peripheral nerve fiber shows Claudin k localising to the mesaxon (*arrow*), Schmidt-Lantermann incisures (*arrowheads*) and paranodal loops (nodes indicated by *asterisk*). D: Along teased single fibers of the spinal cord, Claudin k can similarly be detected in the mesaxon (arrows), Schmidt-Lantermann incisures (arrowheads) and paranodal loops (asterisk). E: Immunolabelling with Claudin k (red) and the tight junction marker ZO-1 (green) in single peripheral nerve fibers shows overlapping localisation in mesaxon (*arrow*), Schmidt-Lantermann incisures (arrowhead) and paranodal loops (asterisk). Scale bars 10µm.

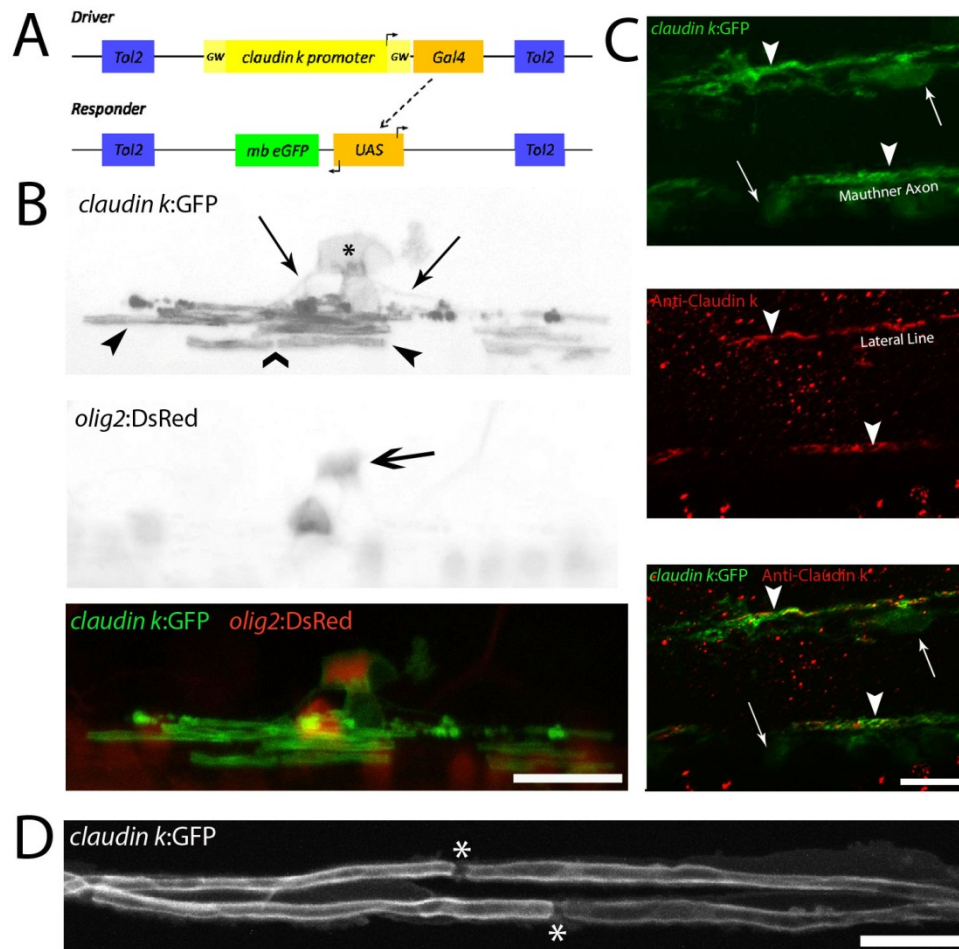


FIGURE 6: Membrane-bound GFP under the control of the *claudin k* promoter strongly labels processes and cell bodies of myelinating glia. A: graphical representation of driver and responder construct for the transgenic *claudin k:GFP*. B: oligodendrocyte in a whole-mounted *claudin k:GFP/olig2:DsRed* double-transgenic zebrafish at 4 dpf (lateral view of the spinal cord). *Claudin k:GFP* expression can be observed in the cell membrane (*asterisk*) and cell processes forming myelin sheaths (*arrowheads*), while *olig2:DsRed* expression (*large arrow*) is only cytoplasmic. The *open arrowhead* indicates a node of Ranvier between two internodes. *Thin arrows* indicate membrane processes of this myelinating cell. C: *Claudin k:GFP* (green) and Claudin k antibody (red) co-label myelin sheaths in the caudal trunk region at 4 dpf (lateral view). Myelin sheaths and oligodendrocyte cell bodies are labeled in *claudin*

*k*:GFP fish, whereas the Claudin *k* antibody only labels myelin sheaths. *Arrows*: GFP positive oligodendrocyte cell bodies. *Arrowheads*: Myelin sheaths. D: A magnification of the caudal part of the PLL in a whole mounted larva at 4 dpf (rostral is left, dorsal is up) nerve shows GFP localisation in the myelin sheaths of two myelinated nerve fibers with nodes of Ranvier (*asterisks*). Scale bars: B = 20µm, C = 20µm, D = 10µm.

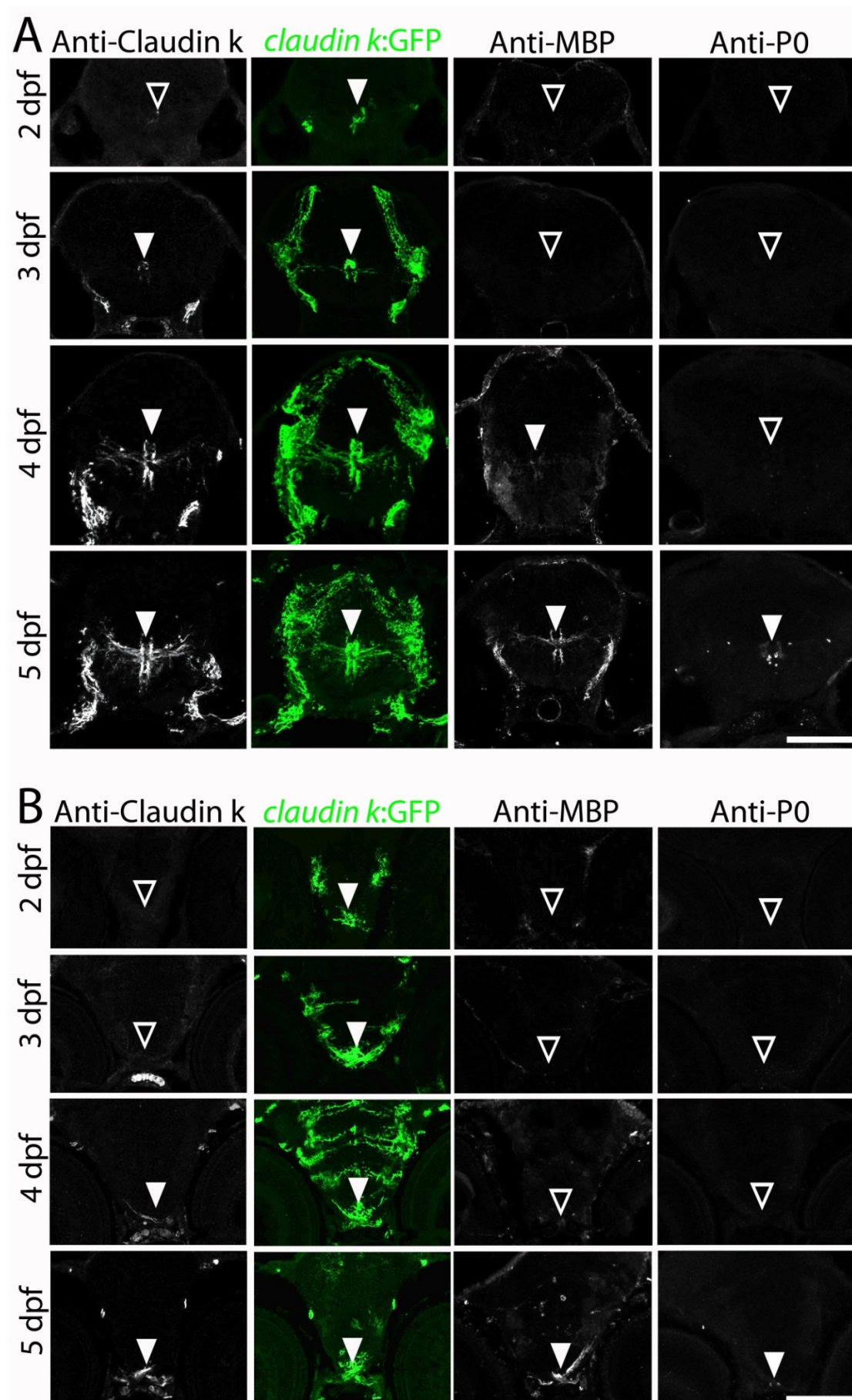


FIGURE 7: Expression timecourse of myelin markers. A: In the hindbrain, myelination in the medial longitudinal fascicle was first observed in the *claudin k*:GFP zebrafish larvae at 2dpf and detection of myelination with antibodies against Claudin k, MBP and P0 were detected 3 - 5 days post fertilization (*full triangles*). *Empty triangles* indicate locations where a GFP signal was observed, but no specific antibody labeling. (Optical ?) cross-sections, dorsal is up. B: In the forebrain in the *claudin k*:GFP zebrafish larvae, myelination in the optic chiasm was also observed first at 2 dpf and evidence of myelin formation using antibodies against Claudin k, MBP and P0 was seen at 4 - 5 dpf (*full triangles*). *Empty triangles* indicate locations where a GFP signal was observed, but no specific antibody labeling. (Optical ?) cross-sections, dorsal is up. Scale bars 100µm.

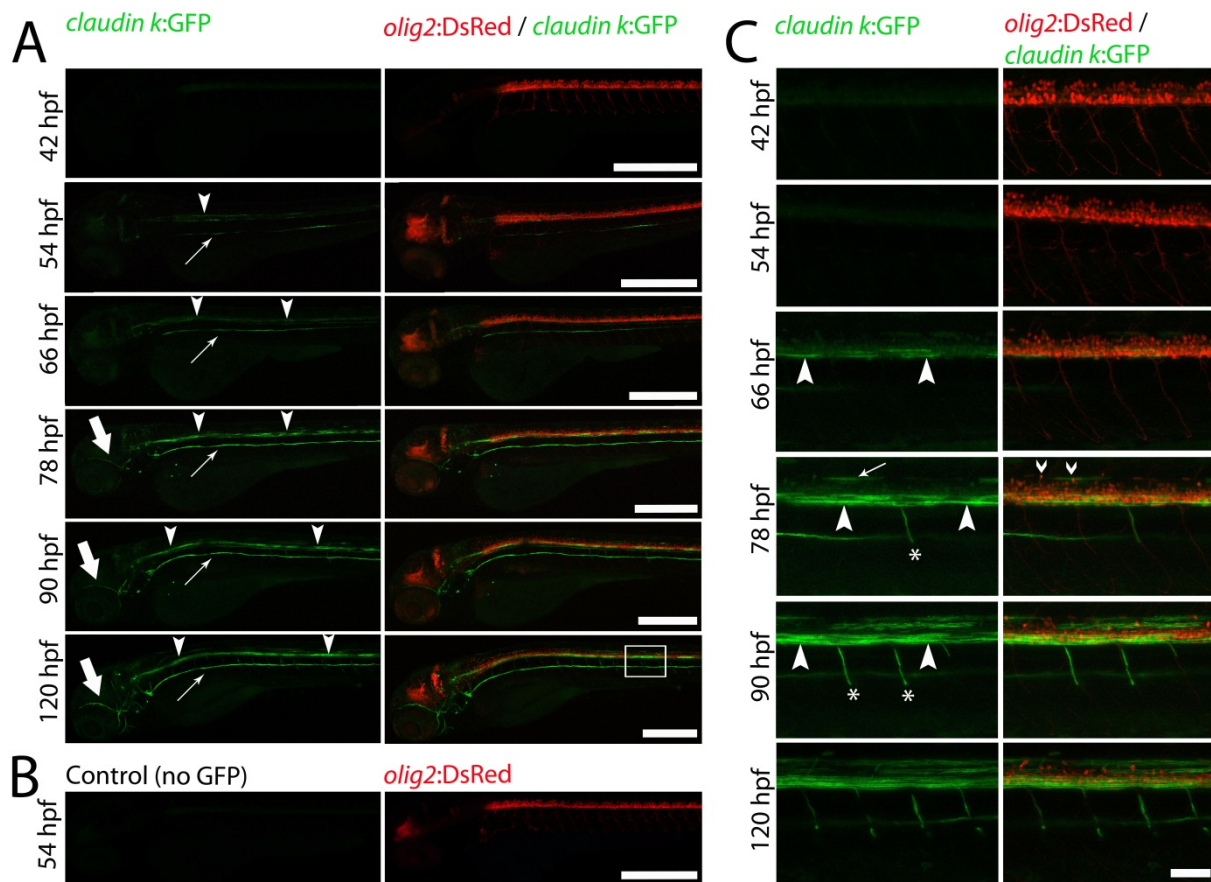


FIGURE 8: Claudin k expression timecourse in a live *claudin k:GFP/ olig2: DsRed* zebrafish larva, lateral view (rostral is left, dorsal is up). A: Transgenic GFP expression was first detected at 54 hpf in the hindbrain and spinal cord (*arrowheads*) and PLL nerve (*thin arrow*). At 78 hpf, additional expression domains can be seen in peripheral nerves such as those innervating ear and eye muscles (*thick arrow*). Myelination progresses from rostral to caudal. B: This GFP expression is not observed in live control larvae. C: A section of the caudal trunk region specified by box in A is shown at higher magnification. GFP expression in the ventral spinal cord can be seen from 66 hpf onwards (*arrowheads*). At 78 hpf we detected myelination of the dorsal longitudinal fascicle of the spinal cord (*arrow*) and of motor axons

(*asterisks*). Dorsally migrating oligodendrocytes were first detected at 78 hpf, (*open arrowheads*). Scale bars: A = 500 $\mu$ m, B = 500  $\mu$ m, C = 100 $\mu$ m.



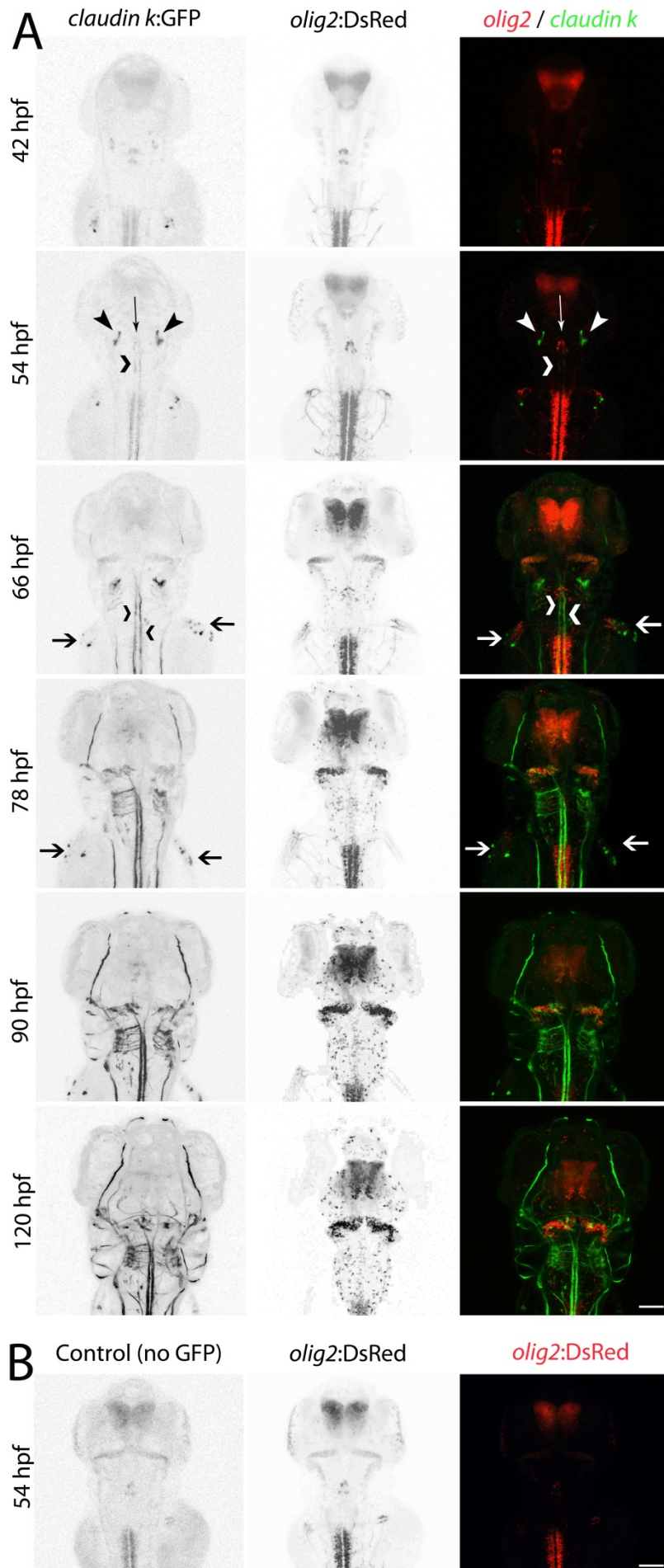




FIGURE 9: Claudin k expression timecourse in a live *claudin k*:GFP/ *olig2*:DsRed zebrafish larva, dorsal view. *Claudin k*:GFP expression can first be seen at 54 hpf in the hindbrain (*arrow*), the medial longitudinal fascicle, structures medial to the otic capsule (*arrowheads*) and the spinal cord (*open arrowhead*). Ectopic GFP expression is present in the fins (*long arrows* at 66 and 78 hpf). B: A single-transgenic *olig2*:DsRed control larva shows no GFP expression. Rostral is up. Scale bars 200µm.

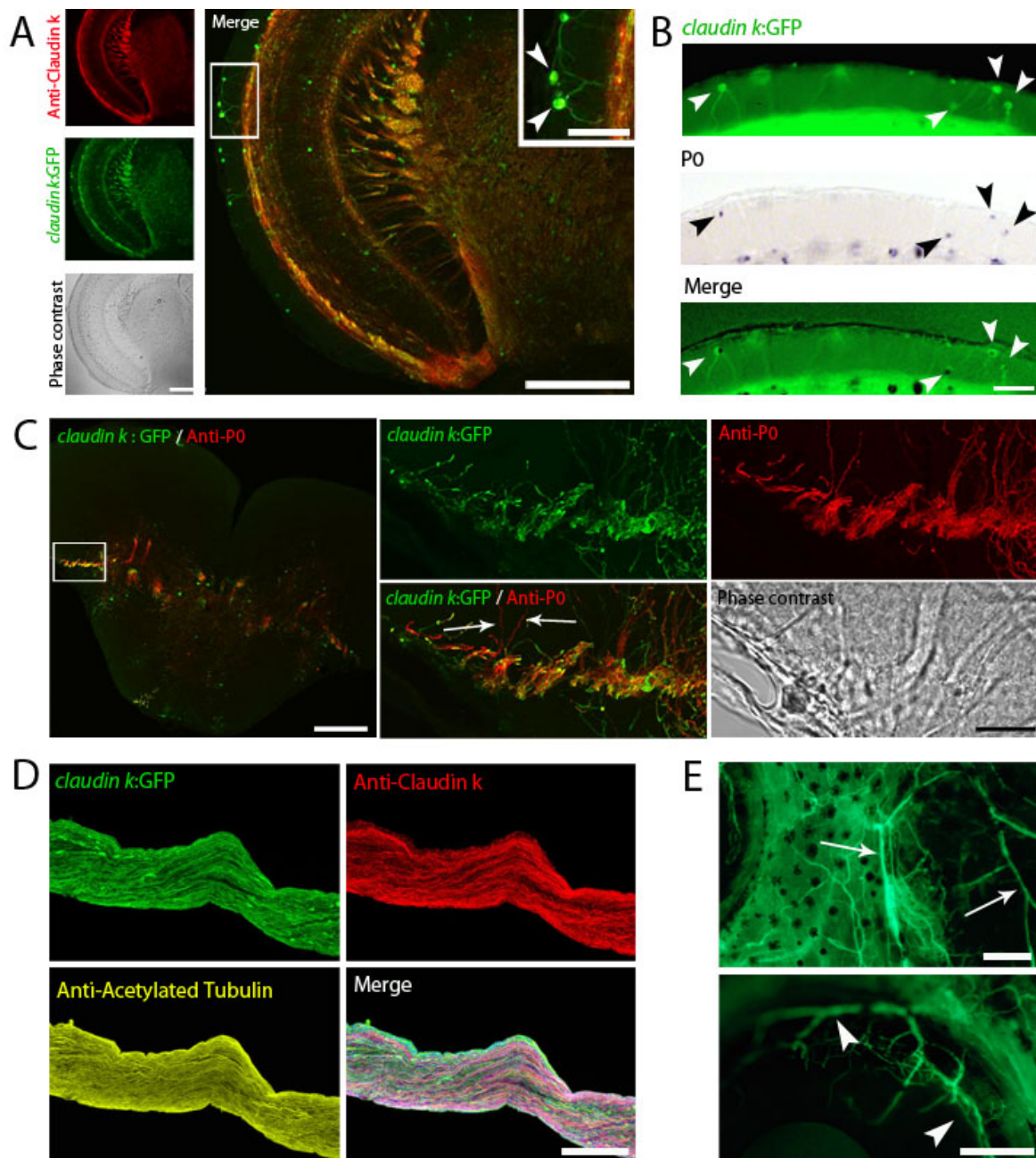


FIGURE 10 Claudin k immunoreactivity and *Claudin k*:GFP expression in the adult zebrafish. A: *Claudin k*:GFP expression in the tectum of adult zebrafish; coronal vibrating blade microtome cross sections. *Claudin k*:GFP-labeled myelin largely overlaps with Claudin k antibody immunoreactivity. *Insert*: GFP-positive oligodendrocyte cell bodies, not labeled by Claudin k antibodies, are shown at higher magnification (*arrowheads*). B: In the tectum, GFP-positive cells are also positive for

P0 mRNA (*arrowheads*), indicating that they are oligodendrocytes. C: *Claudin k*:GFP expression in coronal vibrating blade microtome cross-sections of the telencephalon largely overlaps with anti-P0 immunostaining. Arrows indicate GFP-negative myelin sheaths. D: A whole-mount PLL nerve shows *claudin k*:GFP, anti-Claudin k and acetylated tubulin co-labeling. E: *Claudin k*:GFP expression in the peripheral nerves of skin and eye in anaesthetized live zebrafish under a fluorescent stereo-microscope (rostral is left, dorsal is up). Scale bars: A = 200  $\mu$ m, Insert = 60  $\mu$ m, B = 50  $\mu$ m, C white = 100  $\mu$ m, black 10  $\mu$ m, D = 100  $\mu$ m, E = 0.5 mm.

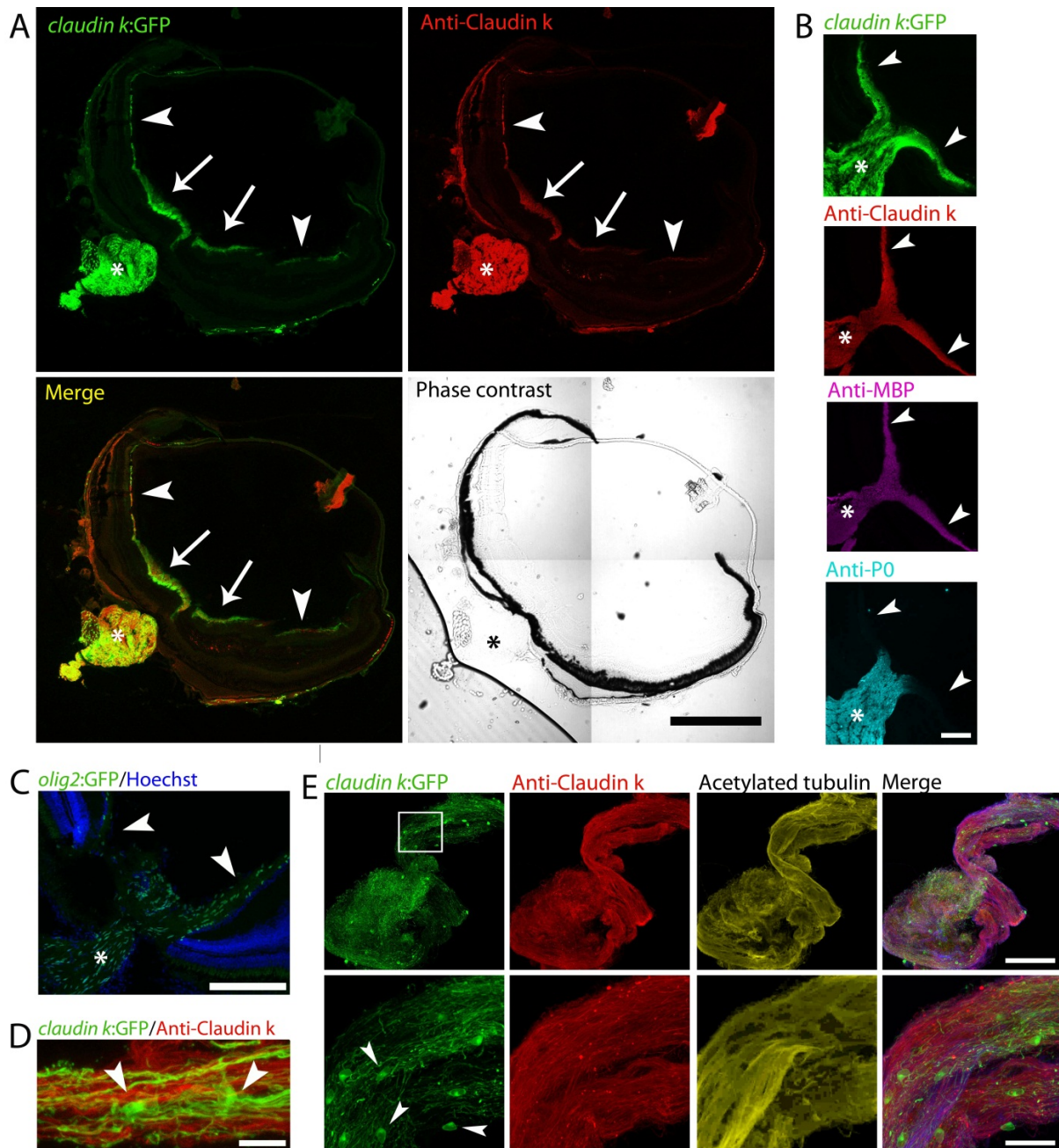


FIGURE 11: Myelination in the visual system of zebrafish. A: Cross section through a zebrafish eye. *Claudin k:GFP* and Claudin k antibody co-label myelin sheaths in the optic axon layer of the eye. *Asterisk*: Optic nerve. *Arrows*: Central retina. *Arrowheads*: Peripheral retina. B: Central retina and optic nerve head are shown at high magnification. P0 is only expressed in the optic nerve and not in the retina (*arrowheads*). *Asterisk*: Optic nerve. C: *Olig2:GFP* positive cells are present in the zebrafish retina (*arrowheads*) and the optic nerve (*asterisk*). D: Oligodendrocytes

expressing GFP in the retina of *claudin k*:GFP fish are shown at high magnification (*arrowheads*). E: Oligodendrocytes (*arrowheads*) can be detected in a whole-mounted optic nerve of a *claudin k*:GFP fish. The boxed area in the top row indicates the area that is shown in higher magnification in the bottom row. Scale bars: A = 500µm, B = 200µm, C = 500µm, D = 10µm, E = 100µm, Insert 40µm.



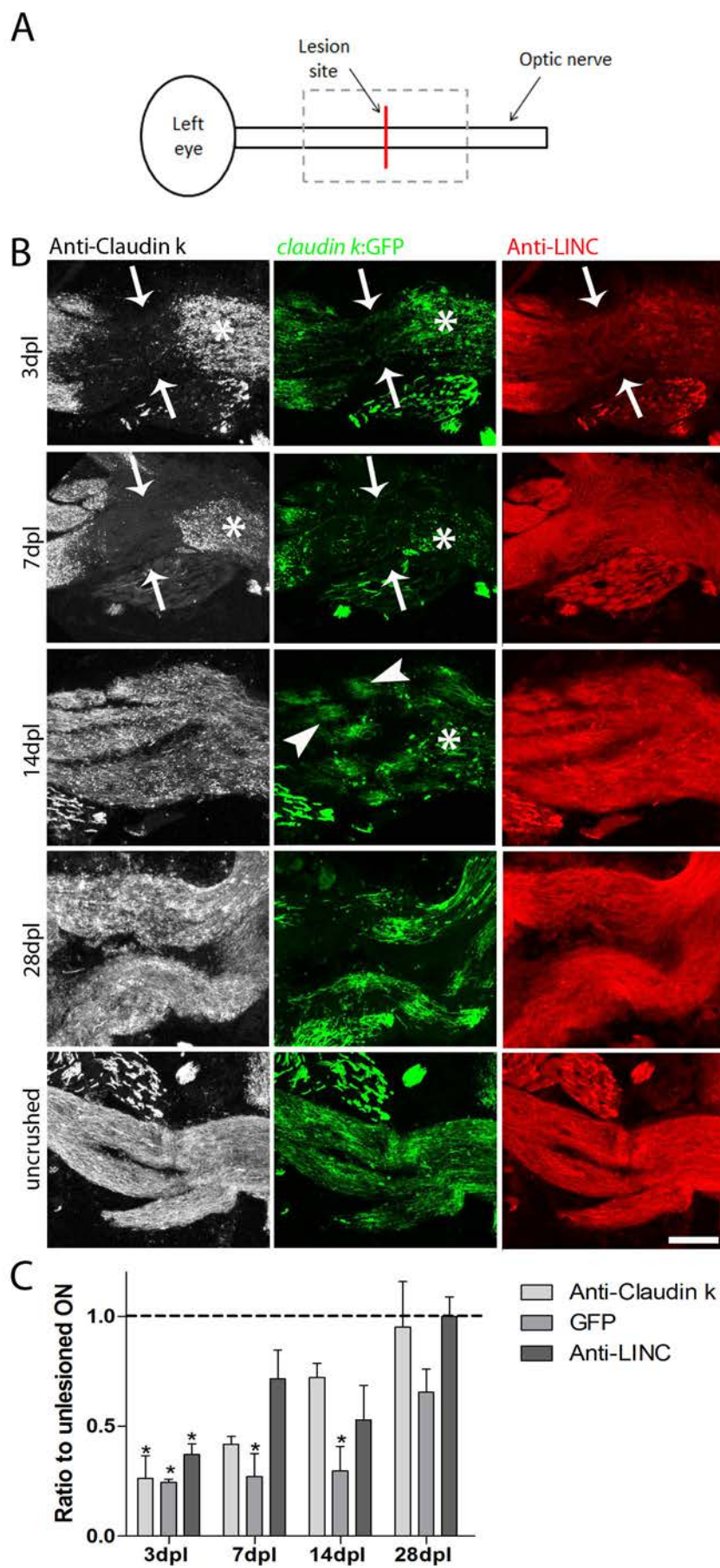


FIGURE 12: Claudin k expression in the crushed adult optic nerve. A: Schematic of optic nerve crush. B: Immuno-labeling of area around lesion (boxed in A). Longitudinal sections of adult zebrafish optic nerves at different post-lesion time points. *Claudin k*: GFP fluorescence and anti-Claudin k and anti-LINC (labeling axons) immunoreactivity in the crush site are decreased at 3 and 7 days post lesion (*arrows*), consistent with Wallerian degeneration. Myelin debris can be observed distal to the lesion site (*asterisks*). By 28 days, GFP fluorescence, anti-Claudin k and anti-LINC immunoreactivity have returned to normal levels in the lesion site, suggesting successful regeneration. Oligodendrocytes with multiple cell processes were detected at 14dpl (*arrowheads*). C: Quantification of immunofluorescence in the lesion site described as a ratio to signal intensity in the contra-lateral unlesioned control nerve. \*  $p \leq 0.05$ . Scale bar: B = 300 $\mu$ m.

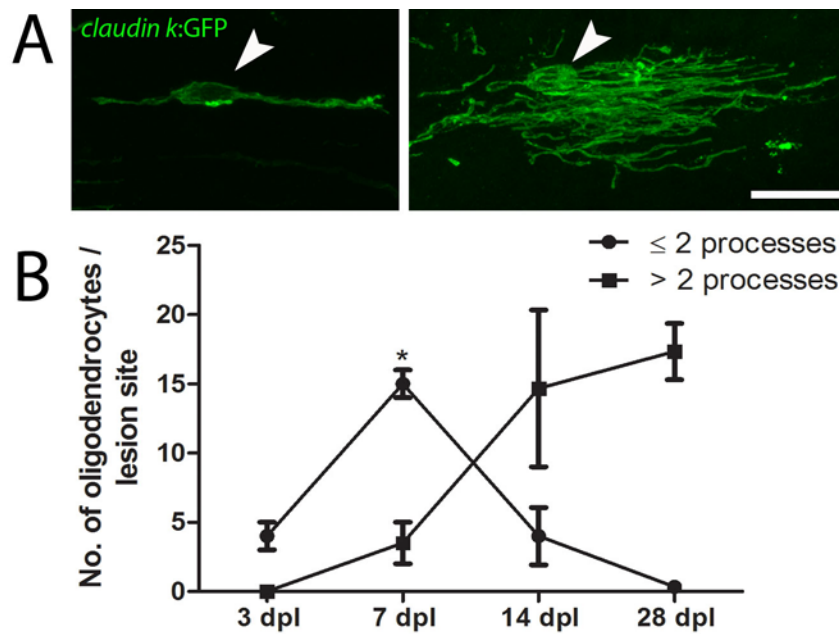


FIGURE 13: Oligodendrocyte morphologies during optic nerve regeneration. A: GFP-expressing oligodendrocytes with various numbers of processes are detected in sections of the lesion site in the adult optic nerve in *claudin k:GFP* transgenic fish at 14 dpl (for orientation see Fig. 12). Cell bodies are indicated by *arrowheads*. B: The number of oligodendrocytes with 2 or fewer processes in the lesion site peaks at 7 dpl. The number of oligodendrocytes with more than 2 processes increases during the regeneration process up to at least 28 dpl. \*  $p < 0.05$ . Scale bar: A = 20 $\mu$ m.